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The Role of Ligand Exchange in the Uptake of Iron from Microbial Siderophores by Gramineous Plants[1,2]

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The siderophore rhizoferrin, produced by the fungus *Rhizopus arrhizus*, was previously found to be as an efficient Fe source as Fe-ethylenediamine-di(*o*-hydroxphenylacetic acid) to strategy I plants. The role of this microbial siderophore in Fe uptake by strategy II plants is the focus of this research. Fe-rhizoferrin was found to be an efficient Fe source for barley (*Hordeum vulgare* L.) and corn (*Zea mays* L.). The mechanisms by which these Gramineae utilize Fe from Fe-rhizoferrin and from other chelators were studied. Fe uptake from ^{59}Fe-rhizoferrin, ^{59}Fe-ferrioxamine B, ^{59}Fe-ethylenediaminetetraacetic acid, and ^{59}Fe-2′-deoxymugineic acid by barley plants grown in nutrient solution at pH 6.0 was examined during periods of high (morning) and low (evening) phytosiderophore release. Uptake and translocation rates from Fe chelates paralleled the diurnal rhythm of phytosiderophore release. In corn, however, similar uptake and translocation rates were observed both in the morning and in the evening. A constant rate of the phytosiderophore's release during 14 h of light was found in the corn cv Alice. The results presented support the hypothesis that Fe from Fe-rhizoferrin is taken up by strategy II plants via an indirect mechanism that involves ligand exchange between the ferrated microbial siderophore and phytosiderophores, which are then taken up by the plant. This hypothesis was verified by in vitro ligand-exchange experiments.

Many agricultural crops worldwide, particularly those in semi-arid climates, suffer from Fe deficiency. The plant kingdom has developed various mechanisms to cope with Fe deficiency (Guerinot and Yi, 1994; Marschner and Römheld, 1994), which include reduction of Fe^{3+}, acidification of the rhizosphere, and release of PS that mobilize Fe from soil minerals. Based on their response to Fe deficiency, higher plants are classified into two strategies (Marschner et al., 1986). Strategy I, prevailing in dicots and non-gramineous monocots, is characterized by a plasma-membrane-bound inducible reductase and an enhanced net excretion of protons. Strategy II is found only in gramineous plants and relies on the release of PS to the rhizosphere and on the induction of a high-affinity system for FePS transport across the plasma membrane. The most common PS are MA, DMA, and epi-hydroxymugineic acid (Kawai et al., 1988). The secretion of PS from Fe-deficient barley and wheat roots was found to occur within a specific period of 4 to 6 h, which usually begins about 2 h after the onset of light (Takagi et al., 1984; Marschner et al., 1986; Zhang et al., 1991). It was later suggested that temperature, rather than a light signal, is the trigger for the initiation of MA release (Mori, 1994); however, it has not been clarified yet whether other plant species share the same diurnal rhythm of PS release.

This classification of higher plants based on their response to Fe deficiency was challenged by Lytle and Jolley (1991). They reported that neither Fe-efficient (WF9) nor Fe-inefficient (Yellow Stripe, YS1) corn cultivars produced sufficient quantities of PS to overcome Fe deficiency. Moreover, corn has also been shown to have some ability to reduce Fe^{3+} to Fe^{2+} at the root plasmalemma (Lytle et al., 1991). In contrast to their findings, von Wirén et al. (1993) found that Alice, an Fe-efficient corn cultivar, does not respond to Fe deficiency via reduction. For the YS1 mutant, von Wirén et al. (1994) were able to show that, although it produces PS, it does not utilize FePS due to a defect in the FePS transport mechanism across the plasma membrane.

The role of MS as chelators that are active in mobilizing Fe to plants that grow under low-Fe conditions in soils has been the focus of many recent investigations. A variety of plant species have the ability to acquire Fe from MS, such as PSB, coprogen, ferrichrome A, and FeFOB (Jurkevitch et al., 1986, 1988; Crowley et al., 1991). Among plant species reported were peanut and cotton (Bar-Ness et al., 1991), oat (Powell et al., 1982; Reid et al., 1984; Crowley et al., 1988), sorghum and sunflower (Cline et al., 1984), and cucumber (Wang et al., 1993). However, the concentrations of siderophores required for a complete remedy of chlorotic plants were higher than those of the synthetic chelators. For example, peanut plants required 10- or 30-fold higher

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Abbreviations: BPDS, (4,7,-diphenyl-1,10-phenanthroline disulfonic acid; CAS, Chrome Azurol S; DFOB, desferrioxamine B; DMA, 2′-deoxymugineic acid; EDDHA, ethylenediamine-di(*o*-hydroxyphenylacetic acid); FOB, ferrioxamine B; HEDTA, hydroxyethylenediaminetriacetic acid; MA, mugineic acid; MS, microbial siderophores; NS, nutrient solution; PS, phytosiderophores; PSB, pseudobactin.

concentrations of FePSB and FeFOB, respectively, than that of FeEDDHA (Jurkevitch et al., 1986, 1988).

The mechanism by which plants utilize MS is not clear. On the basis of their high-stability constants with ferric Fe and their low reduction potential, Bienfait (1989) concluded that MS are not readily reduced by the root plasma membrane reductase system. It was emphasized, however, that kinetics rather than equilibrium (e.g., reduction potential) should be considered (Buyer and Sikora, 1990; Bar-Ness et al., 1991). Wang et al. (1993) found a direct and specific utilization of FeFOB by cucumber plants. Crowley et al. (1988, 1991) reported on the direct and specific utilization of hydroxamate siderophores by oat plants. In contrast, Bar-Ness et al. (1992) and Crowley et al. (1992) showed that Fe supplied as FeFOB to maize plants is mainly taken up by rhizoplane bacteria, which compete with the plants for Fe.

Recently, a new siderophore produced by *Rhizopus arrhizus* has been reported to be an efficient Fe source for strategy I plants, compared with other MS (Shenker et al., 1992, 1995a). In tomato plants the efficiency of Fe-rhizoferrin was similar to that of FeEDDHA. Shenker et al. (1995a) characterized the chemical structure of this siderophore and found it to be chemically identical to rhizoferrin that was produced by *Rhizopus microsporus* (Drechsel et al., 1991).

The present study was designed to investigate the ability of Fe-rhizoferrin to serve as an Fe source for barley (*Hordeum vulgare* L.) and corn (*Zea mays* L.) plants (strategy II), and to elaborate the mechanism by which grasses utilize Fe from MS.

MATERIALS AND METHODS

Plant Culture

Barley (*Hordeum vulgare* L. cv Ma'anit) or corn (*Zea mays* L. cvs Alice, YS1, and Jubilee) seeds were germinated on either quartz sand or filter paper presoaked in a saturated $CaSO_4$ solution. After 4 d the seedlings were transferred to a continuously aerated NS of the following composition: (mM): $Ca(NO_3)_2$, 2.0; K_2SO_4, 0.7; $MgSO_4$, 0.5; KH_2PO_4, 0.1; KCl, 0.1; and micronutrients (μM): H_3BO_3, 10; $MnSO_4$, 0.5; $ZnSO_4$, 0.5; $CuSO_4$, 0.2; $(NH_4)_6Mo_7O_{24}$, 0.01 (Fe-nontreated), or to the same NS with 100 μM FeEDTA (Fe-treated). YS1 plants were precultured for 9 d with 100 μM FeEDTA because of the lower amount of Fe stored in their seeds (von Wirén et al., 1994). All reagents were of analytical grade. Plants were grown in NS for 2 weeks under the following controlled climatic conditions: day/night photoperiod, 16/8 h; light intensity, 220 μE m^{-2} s^{-1} (fluorescent tubes, cool-white FR 96T 12 Sylvania); temperature (day/night) 25/23 ± 1°C; RH 70 to 80%. To improve the Fe status of Fe-treated plants, Fe citrate at a concentration of 0.3% (w/v) was applied as a foliar spray daily, from d 4 prior to the uptake experiment. Fe citrate was prepared by mixing equimolar amounts of $FeCl_3$ and citric acid adjusted to pH 3.7 with NaOH and 0.05% (w/v) X-77 (Loveland, Greely, CO) as a surfactant. To ensure complete prevention of Fe deficiency in some Fe-treated plants, 10 μM FeDMA was applied 1 d prior to the uptake experiment.

Source of Siderophores and PS

Rhizopus arrhizus (strain NRRL 1526) was incubated at 28°C in 5-L flasks containing 500 mL of modified M-9 medium of the following composition (g/L): Glc, 10; Na_2HPO_4, 7; KH_2PO_4, 3; NH_4Cl, 1; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.25; $CaCl_2 \cdot 2H_2O$, 0.015; $ZnCl_2$, 0.015; and thiamine, 0.005 (pH 7.2). After 10 to 14 d the spent medium was collected by filtration, concentrated by freeze-drying, and purified at 4°C through a sequence of three columns: coarse molecular sieving in a Sephadex G-25 column (Pharmacia) eluted with 0.01 M phosphate buffer (pH 7.0), anion exchange through a DE52 column (Whatman) eluted with a gradient of 0.08 to 0.3 M NaCl in 0.01 M phosphate buffer (pH 7.0), and desalting through a Sephadex G-10 column with distilled water. Rhizoferrin was identified by both the light absorbance of its Cu complex at 240 nm and the CAS (Fluka) test (Schwyn and Neilands, 1987), and was quantified by the Cu-CAS test (Shenker et al., 1995b).

DMA was collected as root exudates from corn (cv Alice) roots, and its concentration was determined by HPLC analysis as described elsewhere (von Wirén et al., 1994). MA was obtained from Prof. Satoshi Mori (University of Tokyo, Japan) (it was produced by Fe-deficient barley and purified as described by Mori et al. [1987]). DFOB was obtained as Desferal (Ciba-Geigy, Basle, Switzerland).

Preparation of ^{59}Fe-Labeled Chelates

An aliquot of Fe-free ligand (rhizoferrin, DFOB, DMA, or EDTA) was mixed with $^{59}FeCl_3$ (10% excess ligand), and the pH was adjusted to 6.0 by the addition of 2 mM Mes buffer (Sigma). After the addition of about 5 mL of NS without microelements, the solution was stirred overnight in the presence of filter paper pulp to adsorb Fe colloids and precipitates. The solution was then filtered through a 0.2-μm membrane filter to ensure the removal of non-chelated, colloidal ^{59}Fe-hydroxides. The specific activities were 66.1 GBq mol^{-1} Fe for Fe-rhizoferrin, 148.7 GBq mol^{-1} Fe for FeFOB and FeEDTA, and 16.4 GBq mol^{-1} Fe for FeDMA.

^{59}Fe Uptake Experiments

Roots of barley or corn plants (14–15 d old) were washed for 1 h with microelement-free and aerated NS. The plants were then transferred to beakers containing 140 mL of freshly prepared microelement-free and aerated NS that was buffered to pH 6.0 with 2 mM Mes. The beakers were placed in 500-mL black plastic pots, and ^{59}Fe-labeled chelates were added to provide a final Fe concentration of 10^{-5} M. After 4 h the plants were transferred to a fresh microelement-free and aerated NS for a 10-min wash. After washing the plants were harvested to prevent further translocation to the shoots, and extracellular ^{59}Fe was removed from the roots by 1.2 g/L sodium dithionite and 1.5 mM 2,2'-bipyridyl in 1 mM $CaSO_4$ under N_2 bubbling, according to the method described by Bienfait et al.

(1984). Roots and shoots were oven-dried at 80°C, weighed, ashed at 550°C, and suspended in 1% (w/v) HCl for ^{59}Fe determination by liquid scintillation counting. The following chelators were added to the nutrient solution: (a) barley experiment—^{59}FeDMA, ^{59}Fe-rhizoferrin, ^{59}FeFOB, ^{59}FeEDTA, or ^{59}Fe-rhizoferrin and DMA; and (b) corn experiment—^{59}FeDMA, ^{59}Fe-rhizoferrin, ^{59}FeEDTA, ^{59}Fe- rhizoferrin and DMA, or ^{59}FeEDTA and DMA. The experiments were conducted in the morning and in the evening (2 and 8 h, respectively, after the onset of light) in four replicates, using five or three plants each for barley and corn, respectively. The ^{59}Fe uptake rate for shoots or roots measured as nmol ^{59}Fe is presented per g dry weight of root per 4 h. The Fe translocation rate is presented as ^{59}Fe in shoots per g dry weight per 4 h. Data were subjected to Student-Newman-Keuls analysis (SAS Institute, Cary, NC).

Axenic Plant Culture and Collection of Root Exudates

Corn seeds (cv Alice) were surface-sterilized by immersion for 5 min in 96% (v/v) ethanol and for 20 min in 18% (v/v) H_2O_2, followed by rinsing five times in an autoclaved, saturated $CaSO_4$ solution. The sterilized seeds were germinated on sterile agar plates containing one-half-strength casein-peptone/soymeal-peptone nutrient broth (Merck, Darmstadt, Germany) at 25°C in the dark. After 2 d, axenically germinated seeds were placed into autoclaved culture vessels containing 600 mL of autoclaved NS. Five seedlings were placed in each vessel, and each seedling was held in a pipette tip that allowed the roots to grow downward to the sterile solution. The opening above the autoclaved glass tube was glued by a dental lastic medium (Kettenbach, Kleve, Germany), and the vessels were covered with a black plastic bag. Plants were grown under continuous aeration of the nutrient solution through 0.2-μm filters (Schleicher & Schuell). After 10 d, the upper part of the culture vessel was removed in a laminar flow cabinet, and pipette tips were sealed with sterile cotton wool and 45°C-melted paraffin (congealation point 42–44°C; Merck), so that only the roots continued to grow under axenic conditions. The NS was renewed every 2 or 3 d in a laminar flow cabinet. On d 15 root exudates were collected at intervals of 2 h by replacing the NS with distilled water. Aliquots of the NS were taken after each step to test its sterility via incubation for 2 d in 28°C on nutrient agar medium. Contaminated vessels were discarded, and the experiment was conducted in four replicates of five plants each.

Determination of PS

Micropur (Ag^+ salt; Roth, Karlsruhe, Germany) at a concentration of 0.1% was added to the collected root exudates to prevent microbial degradation of organic solutes during preparation for analysis. Root exudates were then filtered (0.2 μM) and concentrated 60-fold by a Rotavapor (Büchi, Flawill, Switzerland) at 45°C and used for PS determination by HPLC as described by Mori et al. (1987).

Measurement of Fe(III) Reduction Rate

Fifteen-day-old corn plants (cv Jubilee) were transferred to 50-mL Erlenmeyer flasks that were covered with black plastic bags to prevent photoreduction. The flasks contained 20 mL of NS with 0.3 mM BPDS. The pH was adjusted to 5.5 with 5 mM Mes. Fe was supplied as 0.1 mM Fe-rhizoferrin or FeHEDTA (10% excess ligand). Control plants were not supplied with any Fe source. Solutions containing the same composition and chelate, but without a plant, served as a blank for each treatment. Fe^{2+} $(BPDS)_3$ concentration after 1 h was measured by the A_{535} and calculated according to an extinction coefficient of 22.140. The roots were oven-dried at 70°C and weighed. The Fe reduction rate is presented as the amount of Fe^{2+} $(BPDS)_3$ formed in 1 h. The experiment was conducted in four replicates of three plants each. The statistical analysis that was used was the same as that described above.

Ligand-Exchange Experiment

Rhizoferrin, MA, DMA, and their Fe complexes were dissolved in 2 mM $NaHCO_3$ and adjusted to pH 5.5 with NaOH. Based on several preliminary calibration experiments, the standard chelator:Fe ratios were 1:1 for rhizoferrin, and 2:1 for both MA and DMA. The various compounds were separated by TLC, which was performed on a Silica Gel 60 plate (Alugram Sil G/UV_{254} Macherey-Nagel, Düren, Germany) using an ethanol:2 mM $NaHCO_3$ (pH 5.5) 1:1 (v/v) solvent system. Detection of ligands and Fe on the plate was achieved by spraying the plate with a Cu-CAS solution consisting of 880 μM CAS (Fluka), 800 μM $CuCl_2$, and 100 mM Mes (Sigma), pH 5.7 (modified according to Shenker et al., 1995b). Free ligands and Fe chelates were detected as white and blue spots, respectively, on a purple background of the Cu-CAS complex. CAS is a relatively weak chelator of Cu^{2+}, which, upon losing Cu to a competing ligand loses its A_{582}, resulting in a white spot. On the other hand, since the stability constant of CAS with Fe^{3+} is higher than that of Cu^{2+} (Martell and Smith, 1977), Fe^{3+} that is present on the plate will form a spot of the Fe-CAS complex with its characteristic blue color. Since the PS concentration near the active root plane is likely to be higher than that of siderophores, a ratio of 1:2 (MS:PS) was chosen for the ligand-exchange experiment. Fe-rhizoferrin (1:1 ratio) at a concentration of 0.5 mM was incubated with 1 mM MA or DMA for 1 h prior to TLC separation.

RESULTS

Uptake and Translocation by Barley Plants

Data in the literature show that the secretion of PS from Fe-deficient barley roots occurs within a specific period of 4 to 6 h, which usually begins about 2 h after the onset of light (Takagi et al., 1984; Marschner et al., 1986). Fe uptake from Fe-rhizoferrin and other chelates by barley plants was measured in the morning and evening to investigate the influence of PS release on the Fe uptake from ferric chelates. The morning experiments began 2 h after the onset of light, when the release rate of PS is high in barley. The

evening experiments began 8 h after the onset of light, when the release rate of PS is at its lowest daily level.

Uptake and translocation of ^{59}Fe from the various chelates by Fe-deficient plants were generally significantly higher during the morning than in the evening. The differences were most pronounced in plants that were supplied with ^{59}Fe-rhizoferrin. In this case, the evening uptake and translocation rates decreased 1 to 2 orders of magnitude in comparison with their values in the morning (Table I). In plants supplied with ^{59}FeEDTA or ^{59}FeFOB, the same trend was found, but it was less distinct. In fact, during evening hours Fe was not taken up at a substantial rate by the plants from any of the microbial or synthetic chelates.

Addition of free DMA at a concentration of 10 μM, together with ^{59}Fe-rhizoferrin in the evening uptake experiment, significantly improved Fe uptake (about 10-fold) and translocation (about 40-fold) in Fe-deficient plants compared with plants that were only supplied with rhizoferrin (Table I). Translocation rates of plants supplied with ^{59}Fe-rhizoferrin and DMA in the evening were similar to those in plants treated with ^{59}Fe-rhizoferrin in the morning. These results support the hypothesis that Fe uptake from Fe-rhizoferrin is facilitated by PS indirectly via ligand exchange. Fe uptake from ^{59}Fe-rhizoferrin was significantly higher during the morning hours compared with ^{59}FeEDTA and ^{59}FeFOB, but did not approach the Fe uptake rates from ^{59}FeDMA.

In the evening experiment, differences between both uptake and translocation from the various Fe sources, with the exception of ^{59}FeDMA, were much smaller and usually insignificant. This observation is probably the result of a low level of PS release, suggesting a lack or a very low direct utilization of Fe bound to chelators other than PS. In contrast to the Fe uptake and translocation from the microbial or the synthetic chelates, ^{59}Fe uptake and translocation by plants treated with ^{59}FeDMA were high both in the morning and in the evening experiments.

An additional pretreatment with FeDMA was employed before the performance of the morning uptake experiment with a second set of plants that were supplied with ^{59}Fe-rhizoferrin. Under these conditions Fe uptake and translocation from ^{59}Fe-rhizoferrin were significantly lower compared with plants not receiving the additional FeDMA pretreatment.

Uptake and Translocation by Corn Plants

Uptake and translocation of ^{59}Fe from the various chelates by two Fe-efficient corn cultivars, Alice and Jubilee, were similar and much higher than those of the Fe-inefficient mutant YS1 plants (Table II). It should be noted that this mutant has a defect in the PS uptake system (von Wirén et al., 1994). Uptake and translocation of Fe from ^{59}Fe-rhizoferrin by Alice and Jubilee plants were significantly higher than for the FeEDTA-supplied plants (Table II). Addition of 10 μM free DMA to the nutrient solution of Alice plants in the evening or to Jubilee plants in the morning increased Fe uptake and translocation from both Fe-rhizoferrin and FeEDTA. These results are in accordance with the results from the barley Fe uptake experiment, in which Fe uptake from Fe-rhizoferrin and FeEDTA appeared to be indirectly facilitated by PS via ligand exchange. As in barley, uptake and especially translocation of Fe from ^{59}FeDMA by all varieties was significantly higher than from ^{59}Fe-rhizoferrin or ^{59}FeEDTA.

Diurnal Release of PS by Corn Plants

Differences between morning and evening uptake and translocation rates from the various Fe sources in corn were small and usually insignificant (Table II). This finding dif-

Table I. *^{59}Fe uptake and translocation rates from ^{59}Fe-rhizoferrin, ^{59}FeEDTA, ^{59}FeFOB, and ^{59}FeDMA by barley plants grown with and without Fe in the morning and in the evening*

Experimental conditions consisted of: nutrient solution at pH 6.0, 2 mM Mes, 10 μM ^{59}Fe, and 10% excess chelating agent; the uptake period was 4 h, $n = 4$ for each treatment containing 5 plants per pot. Statistical analysis was performed separately for uptake and translocation rates. Different letters represent a significant difference at $P = 0.05$.

Fe Source	Morning Experiment		Evening Experiment	
	Uptake	Translocation	Uptake	Translocation
	nmol g^{-1} root dry wt 4 h^{-1}			
Without Fe				
FeDMA	13,666 a	5,427 a	6,859 b	2,142 b
Fe-rhizoferrin	1,305 c	126 c	48 ghi	5 fgh
Fe-rhizoferrin + DMA	n.d.[a]	n.d.	463 de	189 c
FeFOB	85 g	11 def	34 hij	8 fg
FeEDTA	209 f	23 de	79 g	3 h
With Fe				
FeDMA	9,315 ab	1,911 b	6,116 b	1,822 b
Fe-rhizoferrin	762 cd	135 c	62 gh	5 fgh
Fe-rhizoferrin[b]	287 ef	36 d	n.d.	n.d.
FeFOB	49 ghi	14 def	26 j	12 efg
FeEDTA	83 ghi	18 def	29 ij	4 gh

[a] n.d., Not determined. [b] An additional pretreatment with 10 μM FeDMA was employed 1 d before the morning experiment.

Table II. *^{59}Fe uptake and translocation rates from ^{59}Fe-rhizoferrin, ^{59}FeEDTA, and ^{59}FeDMA by Fe-deficient corn plants in the morning and in the evening*

Experimental conditions consisted of: nutrient solution at pH 6.0, 2 mM Mes, 10 μM ^{59}Fe, and 10% excess chelating agent; the uptake period was 4 h, $n = 4$ for each treatment containing 3 plants per pot. Statistical analysis was performed separately for uptake and translocation rates. Different letters represent a significant difference at P = 0.05.

Cultivar	Morning Experiment		Evening Experiment	
	Uptake	Translocation	Uptake	Translocation
	nmol g^{-1} root dry wt 4 h^{-1}			
Alice				
FeDMA	6,533 b	2,836 a	8,530 ab	3,011 a
Fe-rhizoferrin	692 cd	139 cde	443 ef	193 bcd
Fe-rhizoferrin + DMA	n.d.[a]	n.d.	977 c	465 b
FeEDTA	89 i	9 h	117 hi	33 fg
FeEDTA + DMA	n.d.	n.d.	198 g	75 def
Jubilee				
FeDMA	7,844 ab	3,810 a	10,977 a	4,688 a
Fe-rhizoferrin	515 ef	144 cde		
Fe-rhizoferrin + DMA	890 c	284 bc		
FeEDTA	82 ij	17 gh		
FeEDTA + DMA	137 gh	52 ef		
YS1				
FeDMA	352 f	79 def	561 de	106 def
Fe-rhizoferrin	195 g	2 j	189 g	6 hi
FeEDTA	39 k	2 j	61 j	3 ij

[a] n.d., Not determined.

fers from those found for barley (Table I). The diurnal release of PS from corn plants (cv Alice) was examined. The collection of root exudates from 15-d-old plants revealed a constant exudation rate during the 14 h of growth under light (Fig. 1). This result fits well with the observed high uptake rates in the morning and in the evening (Table II).

Fe^{3+} Reduction by Corn Plants

Since several investigators have reported that corn reduces ferric Fe (Clark and Brown, 1974; Lytle et al., 1991), we examined root ferric reduction to rule out misinterpretation of our uptake data. The reduction rate of Fe^{3+} from rhizoferrin did not significantly differ from that measured on control plants that were not supplied with Fe during the reduction experiment (Table III). In general, the reduction rates measured were about 17- to 54-fold lower than that commonly found in Fe-deficient dicots (Römheld and Kramer, 1983; Römheld and Marschner, 1983; Chaney et al., 1992). Measurements of Fe reduction by roots that were not supplied with Fe-chelate indicate that the low reduction rate measured was actually from the precipitated Fe-hydroxides on the roots, rather than from the chelated Fe^{3+}. The reduction rate of Fe^{3+} chelated by HEDTA was significantly higher than that obtained for Fe-rhizoferrin, although it was not enhanced by Fe stress. Based on these observations we concluded that chelated Fe reduction does not act as an Fe-uptake mechanism of corn plants.

Table III. *Reduction rate of Fe^{3+} from Fe-rhizoferrin and Fe-HEDTA in corn (cv Jubilee) plants compared with control plants not provided with chelated Fe*

Experimental conditions consisted of: nutrient solution at pH 5.5, 5 mM Mes, 100 μM Fe, and 10% excess chelating agent, in the presence of BPDS as the Fe^{2+} chelator; $n = 4$ for each treatment containing 3 plants per pot. Different letters represent a significant difference at P = 0.05.

Fe^{3+} Source	Fe^{3+} Reduction	
	With Fe	Without Fe
	nmol Fe^{2+} g^{-1} root dry wt h^{-1}	
No Fe source	488.1 bc	212.3 d
Fe-rhizoferrin	366.8 cd	417.1 cd
FeHEDTA	915.7 a	691.7 ab

Table IV. *Demonstration of ligand exchange between Fe-rhizoferrin and phytosiderophores by TLC separation and color development with a Cu-CAS reagent*

TLC was performed on a Silica Gel 60 plate using ethanol and a 2 mM $NaHCO_3$ (pH 5.5) mixture at a ratio of 1:1 (v/v) as a solvent system. For ligand-exchange experiments Fe-rhizoferrin (1:1) was incubated with MA or DMA at a ratio of 1:2 for 1 h prior to TLC separation.

Sample	Blue Spot (Fe complex)	White Spot (free ligand)
	R_F	
Standards		
Rhizoferrin	n.c.[a]	0.81
Fe-rhizoferrin	0.87	n.c.
MA	n.c.	0.84
FeMA	0.16	0.85
DMA	n.c.	0.73
FeDMA	0.19	0.72
Ligand-exchange experiment		
Fe-rhizoferrin + MA (1:2)	0.16, 0.86	0.79
Fe-rhizoferrin + DMA (1:2)	0.18, 0.74	0.69

[a] n.c., No color.

In Vitro Ligand Exchange

The demonstration of ligand exchange in vitro is complicated, since both rhizoferrin and PS and their Fe complexes are colorless. Thus, a new visualization TLC procedure has been developed for the separation of the various compounds based on blue color development with a Cu-CAS reagent upon exchange with FeMS. The results of this experiment (Table IV) clearly show the formation of FeMA and FeDMA after incubation of the free PS with Fe-rhizoferrin. This is evident from the appearance of blue spots in the characteristic R_F values of 0.16 and 0.18 for FeMA and FeDMA, respectively. These data provide direct evidence of the involvement of ligand exchange in the Fe-uptake mechanism from Fe-siderophores.

DISCUSSION

MS investigated to date, excluding rhizoferrin, have exhibited low efficiency as Fe carriers to higher plants (Jurkevitch et al., 1986, 1988; Bar-Ness et al., 1992; Crowley et al., 1992). However, for strategy I plants, rhizoferrin, a siderophore produced by *R. arrhizus*, has been reported to be a very efficient source of Fe (Shenker et al., 1992, 1995a). For strategy II plants, PS are essential to ensure sufficient Fe supply. The preference of Fe uptake from FeDMA for both barley and corn plants shown in this paper are in accordance with previous studies that were conducted on Fe uptake in gramineous plants (Römheld and Marschner, 1986). ^{59}Fe uptake and translocation rates from both FePSB in corn and FeFOB in oat were 2 and 3 orders of magnitude lower than from FeHMA, respectively (Bar-Ness et al., 1992); the same ratios were reported by Crowley et al. (1992). Nevertheless, compared with FePSB and FeFOB, rhizoferrin showed a relatively high efficiency in providing Fe to barley and corn. Fe uptake and translocation rates were only 1 order of magnitude lower than those obtained for FeDMA. We concluded from these observations that the Fe-rhizoferrin provides a realistic tool for studying the Fe uptake mechanism from MS by Gramineae.

We propose that a substantial amount of Fe from MS is taken up as FePS by barley, corn, and probably also by other gramineous plants after ligand exchange in the solution. The rate of this reaction and its effectiveness in supplying Fe from MS to plants is controlled by the following factors: (a) amount of PS released, (b) concentration of the FeMS, (c) stability constants of the various ligands involved with Fe^{3+} and other competitive metals, (d) kinetics of ligand exchange between Fe-siderophore and the free PS, and (e) efficiency of the FePS uptake system. A similar concept for the interactions between organisms in relation to Fe nutrition was described by Buyer and Sikora (1990), and in the present work we studied some of these aspects.

The influence of PS concentrations in the medium on Fe uptake from Fe chelates by barley and corn was demonstrated by the enhancement in Fe uptake and translocation rates that resulted from external DMA additions to the solution (Tables I and II). The high uptake and translocation rates of Fe by barley in the morning, compared with the evening, provide additional support for the involvement of MA or DMA in the Fe uptake process. This is attributed to the high secretion of PS during that period (Takagi et al., 1984; Marschner et al. 1986). The same diurnal rhythm of PS release by Fe-deficient or Zn-deficient plants has been reported for wheat (Zhang et al., 1991). When FeDMA was provided as an Fe source to corn plants, the translocation rate was equal in the morning and in the evening. These results are supported by the observation that PS are released in corn at a constant rate during 14 h of light. This trend held even though FeDMA was applied in the evening. The results obtained by Mihashi et al. (1991) could provide an explanation. They found that FeMA at an Fe:MA ratio near 1 may dissociate, and Fe may be adsorbed to phospholipids on the cell surface. However, at the excess of MA this reaction does not occur.

The similarity of morning and evening Fe uptake and translocation rates by corn plants, as measured in this study, seems to contradict reported data on PS secretion by gramineous plants. Although information on the diurnal rhythm of PS release by corn plants was not available, all Gramineae were assumed to behave similarly (Marschner et al., 1986; Bienfait, 1988). Examination of the PS release during 14 h of growth under light revealed a constant secretion rate (Fig. 1). This pattern of uniform PS release rate fits well with the Fe uptake and translocation rates measured in the morning and evening experiments (Table II). The close agreement between the PS release patterns of corn and barley with their uptake and translocation patterns further support our hypothesis that ligand exchange is essential to Fe utilization from MS.

The second component of Fe uptake in strategy II plants is the membrane-bound receptors for FePS transport into the plant cell. von Wirén et al. (1994) have shown that the corn mutant YS1 has a defect in the FePS uptake system. Fe uptake and especially translocation from FeDMA, Fe-rhizoferrin, or FeEDTA was 30- to 50-fold lower than in Alice or Jubilee (Table II), suggesting that in the absence of an efficient FePS uptake system there is no alternative Fe uptake pathway from microbial or synthetic chelates in gramineous plants.

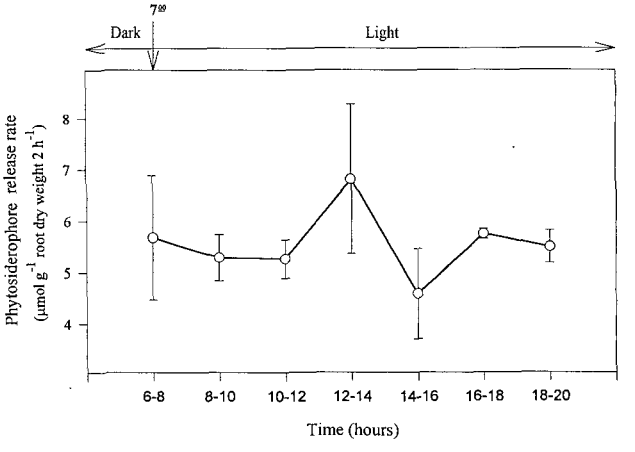

Figure 1. Diurnal release of phytosiderophores by 15-d-old Fe-deficient corn (cv Alice) plants grown under sterile conditions.

The enhancement of Fe uptake and translocation from Fe-rhizoferrin in the presence of free DMA, and the high uptake and translocation from FeDMA in the evening, indicate that the factor limiting Fe uptake in the evening is the lack of PS release and not the uptake system of FePS. In Fe-sufficient plants supplied with FeDMA, uptake and translocation rates of Fe were high, demonstrating that uptake of ferrated PS is affected by Fe deficiency to a lower degree than PS release. This was also shown by von Wirén et al. (1994). Therefore, it is concluded that under Fe deficiency, gramineous plants such as barley secrete enhanced levels of PS during the morning hours, whereas the uptake system operates continuously.

The differences between the efficiency of rhizoferrin, EDTA, and FOB in supplying Fe to barley plants can be explained by differences in their apparent stability constants (K_{app}) with Fe^{3+}. K_{app} as described in detail by Shenker et al. (1996) is a pH-dependent constant, and for the reaction

$$Fe^{3+} + L_{total} \Leftrightarrow FeL_{total}$$

is defined as:

$$K_{app} = \frac{[FeL_{total}]}{[Fe^{3+}][L_{total}]}$$

where L_{total} and FeL_{total} represent the sums of all protonation species of the ligand of its Fe complex, respectively, and brackets represent molar concentrations. K_{app} values were calculated according to stability constants that are reported in the following references: DFOB, data base of Geochem-PC version 2.0 (Parker et al., 1995); EDTA, Martell and Smith (1974); rhizoferrin, Shenker et al. (1996), and MA and DMA, Murakami et al. (1989). Calculation of the apparent stability constants of the studied chelates with Fe^{3+} at pH 6.0, 25°C, and 0.1 M ionic strength resulted in K_{app} values of 21.2, 20.3, 17.1, 15.7, and 15.3 for DFOB, EDTA, rhizoferrin, DMA, and MA, respectively. Therefore, ligand exchange between Fe-rhizoferrin and free PS in excess is expected. However, K_{app} values for FeEDTA and FeFOB are too high to allow effective ligand exchange. Direct evidence for the existence of such a ligand exchange between PS and rhizoferrin was provided using a new visualization TLC procedure based on color development with a Cu-CAS reagent (Table IV).

A ligand-exchange reaction of MS (PSB) and MA has been previously demonstrated (Jurkevitch et al., 1993). PSB has a K_{app} of 22.5 at pH 6.0, 25°C, and 0.1 M ionic strength (Chen et al., 1994). It was found that ligand exchange from FePSB to MA did not occur at all, whereas a complete exchange from FeMA to PSB occurred with a half-life of 2 h (Jurkevitch et al., 1993).

According to the above values for K_{app}, our calculations show that a solution of a 1:1:1 ratio of rhizoferrin, MA, and Fe^{3+} is expected to equilibrate at about 90% of the rhizoferrin as Fe-rhizoferrin and about 10% of MA as FeMA. At any specific time before equilibrium is reached the level of FeMA may be lower than 10%, depending on kinetic considerations. However, the uptake rate of FeMA is by far faster and more specific than that of Fe-rhizoferrin, which was shown in this work. A sink for FeMA will divert the ligand-exchange reaction to the formation of more FeMA. Moreover, as shown by Crowley and Gries (1994) the concentration of PS in the rhizosphere at the root tips during the production period is extremely high and exceeds the concentration of MS that might be expected in that location.

The possibility of additional Fe-uptake mechanisms (e.g. reduction of Fe^{3+} operating in corn) was suggested by Lytle and Jolley (1991). Results obtained from the present study show that Fe^{3+} reduction from Fe-rhizoferrin operates at an extremely low level. Fe^{3+} reduction rates from both Fe-rhizoferrin and FeHEDTA by Fe-deficient and Fe-sufficient corn plants indicate that the reduction is not induced by Fe stress (Table III). These results are in accordance with those of von Wirén et al. (1993), who showed that reduction, acidification, and release of reducing compounds in corn (cvs WF9 and YS1) are not influenced by Fe deficiency. These results are also in agreement with Bar-Ness et al. (1992), who showed that the addition of BPDS to solutions did not inhibit Fe uptake or translocation from FePSB and FeHMA by corn plants, thus excluding the possibility of Fe^{3+} reduction playing a significant role prior to uptake of chelated Fe. The reduction that did occur might be attributed to the "basic system" that is not influenced by the Fe status of the plant as suggested by Bienfait (1985). This system operates in plants using either strategy I or II, and it is most likely used to export protons that are needed to build a transmembrane potential.

Evidence provided in this paper demonstrates the essential role of PS in Fe uptake by strategy II plants, as well as the possible importance of exchange of Fe from MS to PS in Fe uptake. Ferric complexes of MS and synthetic chelates, with low rates of ligand exchange of the Fe with free PS, are poor Fe sources to Gramineae, whereas Fe-rhizoferrin was found to efficiently provide Fe via ligand exchange.

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